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Original Article

Deciphering the involvement of iron targets in colorectal cancer: a network biology approach

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Abstract: Several studies suggested the role of heme iron, but not non-heme iron in colorectal cancer. A network and system biology-based approach was used to understand the role of heme and non-heme iron on colorectal cancer etiology. Heme and non-heme iron targets were screened in addition to CRC targets. The protein-protein interaction map of both iron targets was prepared with CRC targets. Moreover, functional enrichment analysis was performed in order to understand their role in cancer etiology. The heme iron is predicted to modulate several cancer-associated pathways. Our results indicate several targets and pathways, including IL-4/IL-13, ACE, and HIF-1 signaling, that may have an important role in heme iron-mediated CRC and must be given consideration for understanding their role in colorectal cancer.

Keywords: Heme, cancer biology, carcinogenesis, colon cancer, signaling, protein-protein interactions

Introduction

Colorectal cancer (CRC) is considered as the 4th most commonly diagnosed cancer, which is also ranked 3rd for cancer-associated death according to GLOBOCAN, 2018 data [1]. The cases of CRC are generally attributed to certain risk factors categorized as modifiable and non-modifiable risk factors. These factors include host genetics, certain components of host microflora, smoking, dietary habits, physical inactivity, overweight, etc. [2-5]. The role of iron in CRC etiology has been suggested in several studies ranging from its involvements to no association. Iron is an important micronutrient involved in several biological processes including immune health, RBC development, and its deficiency is recognized as the most common cause of anemia [6]. Colorectal cancer patients are also often considered to be anemic due to chronic tumor mediated blood loss, impaired iron homeostasis, and post-operative complications [7]. Therefore, iron therapy is recommended, but this may also support CRC and increases its metastasis [8]. Some epidemiological investigations have also reported positive correlation of iron with CRC [9, 10].

Iron is generally available in the form of heme or non-heme iron and both of these differ in several ways. Heme iron is present in animal food, such as red meat and it is bound to heme proteins and is absorbed relatively easy in digestive tract. In contrast, non-heme iron is found in plant-based food, which is present as either Fe(II) and Fe(III). Fe(II) also oxidizes to Fe(III) and either precipitates or forms soluble hydroxyl-iron dimers making its absorption comparatively difficult [11]. The intake of red meat is associated with high risk of colon cancer and partly this is credited to the heme iron present in the meat. It is suggested that heme produces certain factors, such as cytotoxic heme factor, leading to hyperproliferation and subsequent risk of colon cancer [12, 13]. On the other hand, some studies have found negative or no association of iron with colorectal cancer risk [14].

We performed this study in order to understand how heme and non-heme iron can affect CRC etiology. We screened heme and non-heme iron targets and their subsequent interactions with colorectal cancer targets. Further, we also assessed how these targets can modulate necessary biological processes and pathways related to colorectal cancer. We explored the influence of heme iron on CRC and present here certain targets to devise strategy for prevention of CRC modulating effects of heme.

Materials and methods

Target databases

The most abundant heme iron protoporphyrin IX was used to screen heme targets while ferric citrate and ferrous fumarate were screened to identify non-heme targets. These molecules are also used as therapeutic agents to manage anemia and this was another rationale to select these molecules as representative of heme and non-heme iron source. The targets of heme and non heme iron were screened at DrugBank (version 5.1.7, released 2020-07-02) [15], Swiss Target Prediction [16], The Binding Database [17], DGIdb (v3.0.2 last updated 25/01/2018) [18], Super Target [19], and Therapeutic Target Database 2020 [20]. All the target databases were screened on or before 18 August 2020. Moreover, the targets of colorectal cancer were identified at DisGeNet (CUI: C0009402) [21].

Screening of heme and non-heme targets involved in colorectal carcinoma

The Venn diagram was constructed for all the analyzed heme and non-heme iron targets with CRC targets. The CRC targets influenced by heme and non-heme iron were screened for further analysis. In addition, the common targets among heme and non-heme iron were also analyzed in order to study targets commonly or exclusively influenced by heme and non-heme iron.

Functional identification and protein-protein interaction network construction

The function of heme and non-heme iron targets was identified from Uniprot and their role in CRC was collected through web search. The protein-protein interaction network of iron targets and CRC-associated human proteins (as per DisGeNet) was constructed through STRING. Cytoscape v. 3.8 was used to construct and visualize interactions and topological parameters of network nodes were estimated using network analyzer. The STRING was also used for functional enrichment of heme and non-heme targets and their respective CRC specific interactors as per DisGeNet.

Functional over-representation analysis of heme targets and their CRC-associated first neighbors

Earlier step showed that the heme targets are involved in modulation of pathways of cancer, while non-heme targets were not enriched in such process. Therefore, heme targets and their direct CRC associated interacting proteins identified during protein-protein interaction map through STRING were further analyzed for evaluating their functional overrepresentation. Selected Heme targets and their first neighbors subnetwork was prepared through cytoscape and analyzed for functional overrepresentation analysis using g:Profiler.

Results

Screening of heme, non-heme, and colorectal cancer targets

A total of 12, 15, and 13 targets were screened from 6 drug target databases for protoporphyrin IX, ferric citrate and ferrous fumarate respectively. Out of total 12 heme targets, 2 were identified from non-human sources and 1 was repeated in multiple databases; therefore, a total of 9 human-specific protein targets were identified. For the 15 targets of ferric citrate, 2 were identified from non-human sources and 1 was repeated in multiple databases, giving 12 human-specific unique protein targets.

A total of 5473 targets were obtained for colorectal carcinoma in DisGeNet database. The uniprot ID details of only 4793 targets were available out of 5473 as some other entries, such as miRNA etc. were also included in disease target database, and used for further analysis after ID mapping.

Screening of heme and non-heme targets involved in colorectal cancer

The heme and non-heme targets involved in influencing CRC are presented in **Table 1**. No

common screened target was found between heme and non-heme during our study. However, the ferric citrate and ferrous fumarate were having 2 common human protein targets. **Table 1** indicates heme and non-heme iron targets and the targets involved in CRC, such as STAT3 and Transferrin receptor protein 1. In addition, the targets common between ferrous fumarate and ferric citrate are also presented.

Functional identification and protein-protein interaction network construction of heme and non-heme iron targets

The function of heme and non-heme targets as per Uniprot in addition to their CRC-specific function is presented in **Tables 2** and **3**, respectively. The protein-protein interaction network of selected CRC specific heme iron targets is presented in **Figure 1**. The functional over-representation analysis results of top ten hits of heme and non heme targets and their CRC specific interactors (according to STRING enrichment) are presented in **Figure 2**.

Functional enrichment of heme targets and their direct CRC-associated interactors

As the heme targets were identified to modulate cancer-associated pathways, these targets were further analyzed separately in order to assess their role in more specific processes. The detail about g:Profiler top hits with gene ontology (biological process) and biological pathway modulation (KEGG, Reactome, and WikiPathways) are presented in **Figure 3** on the basis of adjusted *P*-value.

Discussion

CRC development is linked to several modifiable factors. High consumption of red and processed meat is one of these factors. *In vivo* experimental evidences have indicated that hemoglobin and red meat consistently promote aberrant crypt foci leading to precancerous changes. Although the exact mechanisms are not known, it is suggested that heme iron induces carcinogenesis through formation of N-nitroso compounds and aldehydes (through lipid peroxidation) [22]. In contrast, some studies have indicated no association of body iron with increased CRC but suggested its role in combination with high fat diet [23]. Therefore, it is pertinent to understand the role of heme and non-heme iron in CRC.

We used network pharmacology-based approach to understand heme and non-heme targets and their interactors involved in CRC. The network pharmacology is a relatively new concept, which is far ahead of one molecule and one target concept. It works to identify multiple target components and their interrelations using network biology, and have been used in several cancer-related studies [24-27]. We used standard and widely accepted databases for screening of heme and non-heme targets, which screen target proteins on the basis of variety of criteria. Certain targets were redundant in different databases while sometimes they are also mentioned with literature evidences indicating additional reliability of screening results. The DisGeNet is also a widely accepted database of disease associated genes. It is claimed to be the largest database of disease associated genes, containing data taken from curated repositories, scientific literature, GWAS catalogues, and animal models. It consists of 1,134,942 disease-gene associations between 30,170 disease and 21,671 genes at the time of analysis [21, 28]. **Table 1** indicates heme and non-heme targets involved in CRC, such as STAT3, Renin, Transferrin receptor protein 1, Ferritin heavy chain, etc. The results found no common targets between heme and non heme iron, which indicate that both the irons may act differentially in modulating CRC. We performed protein-protein interactions analysis of iron targets with CRC target using database STRING which is also a well-known database of protein-protein interactions and includes both the direct and indirect associations [29].

Both heme and non-heme iron targets were found to interact with CRC targets. Therefore, we considered separate functional enrichment analysis of heme and non-heme iron targets and their interactors in order to assess their role in CRC. The non-heme targets of ferrous fumarate did not show overrepresentation of cancer-associated term in top 200 enriched processes according to FDR-value. Similarly, non heme iron ferric citrate did not show cancer-associated term in top 50 enriched processes. In contrast, heme iron showed enriched process “pathways in cancer” among top 10 pathways. Therefore it indicates that heme iron

Table 1. Screened heme and non-heme iron targets (human proteins) and their involvement in CRC

Protoporphyrin IX	Uniprot ID	Ferric citrate	Uniprot ID	Ferrous fumarate	Uniprot ID
Ferritin light chain	P02792	Transferrin receptor protein 1	P02786	Transferrin receptor protein 1	P02786
Translocator protein	P30536	Cytochrome b reductase 1	Q53TN4	Egl nine homolog 1	Q9GZT9
Pepsin A	P0DJD9	Serotransferrin	P02787	Serotransferrin	P02787
Renin (by homology)	P00797	Integrin beta-3	P05106	Alpha-hemoglobin-stabilizing protein	Q9NZD4
Telomerase reverse transcriptase	O14746	Calreticulin	P27797	Hemoglobin subunit alpha	P69905
Signal transducer and activator of transcription 3	P40763	Natural resistance-associated macrophage protein 2	P49281	Frataxin, mitochondrial	Q16595
Autotaxin	Q13822	Solute carrier family 40 member 1	Q9NP59	Ferritin heavy chain	P02794
Hematopoietic prostaglandin D synthase	O60760	ATP-citrate synthase	P53396	Flap endonuclease 1	P39748
Prostaglandin G/H synthase 1	P23219	Neutrophil cytosol factor 2	P19878	Endonuclease 8-like 1	Q96FI4
		SH2 domain-containing protein 1A	O60880	Endonuclease 8-like 2	Q969S2
		Signaling lymphocytic activation molecule	Q13291	DNA polymerase beta	P06746
		Urokinase-type plasminogen activator	P00749	Ceruloplasmin	P00450
				Histone deacetylase 8	Q9BY41

Red color entries indicate proteins involved in CRC according to DisGeNet.

Table 2. Function of human protein targets interacting with heme and also involved in CRC

Protoporphyrin IX (heme) targets	Function from Uniprot	Role in cancer
Ferritin light chain (P02792)	Involved in iron homeostasis, delivery and storage in non-toxic and readily available form.	Upregulated in CRC tissues and cell lines and promote chemoresitance and metastasis [51].
Translocator protein (P30536)	Transport porphyrin, heme, and cholesterol.	Involved in cellular proliferation, apoptosis and mitochondrial function. Expression level increased in colon cancer but not in rectal cancer [52].
Renin (P00797)	Endopeptidase generating angiotensin I from angiotensinogen. Elevate blood pressure and sodium retention by kidney.	Pro (renin) receptor is involved in promotion of CRC through Wnt/beta-catenin signaling [53].
Telomerase reverse transcriptase (O14746)	Involved in replication of chromosome termini, but active in progenitor or cancer cells. Elongate the telomerase by adding sequence repeats to chromosome ends. Modulate Wnt signaling and involved in apoptosis inhibition and aging.	Overexpression is associated with migration and proliferation of CRC cells [54].
Signal transducer and activator of transcription 3 (P40763)	Respond to several growth factors. Promote various acute phase protein genes through IL-6 signaling [55]. Regulate inflammatory response, cell cycle, and apoptosis.	STAT3 expression is associated with host inflammatory response and decreased survival in patients undergoing CRC resection [56].
Hematopoietic prostaglandin D synthase (O60760)	Bifunctional enzyme leading to conversion of PGH2 to PGD2 (prostaglandins). Inhibit platelet aggregation, and glutathione conjugation with aryl halides and organic isothiocyanates.	It can suppress intestinal adenomas in ApcMin/+ mice [57].
Prostaglandin G/H synthase 1 (P23219)	Involved in prostanoid production through conversion of arachidonate to PGH2, specially in gastric cells. Involved in platelet activation, aggregation and vasoconstriction through generation of thromboxane A2.	Polymorphisms in PTGS1 may increase CRC risk [58].

Table 3. Function of human protein targets interacting with non-heme iron and also involved in CRC

Ferric citrate/Ferrous fumarate (non heme) targets	Function from Uniprot	Role in cancer
Transferrin receptor protein 1 (P02786)	Involved in cellular uptake of iron, erythrocyte development and nervous system development, T and B cell proliferation. Involved in JNK pathway regulation.	Negative regulation through Mir107 leads to supression of CRC cell proliferation, migration and invasion of SW620 cells [59].
Cytochrome b reductase 1 (Q53TN4)	Reduces Fe3+ and fecilitate its trasnport to mucosal cell in duodenal enterocytes, possibly mediate ascorbate recyling in erythrocyte membrane, acts as ferrireductase in epithelial cells of airway.	Polymorphism is associated with CRC using susceptibility gene-ancestry interactions statistical method on genome wide association study data [60].
Integrin beta-3 (P05106)	Acts as receptor for several molecules and microbes.	Involved in epigenetic and transcriptional regulation contributing to CRC carcinogenesis and progression [61].
Calreticulin (P27797)	Calcium binding chaperone. Regulate ER, interact to DNA binding domain of NR3C1 and control its nuclear export.	miR-27a mediated MHC class I repression through calreticulin downregulation modulate CRC tumor progression [62].
Natural resistance-associated macrophage protein 2 (P49281)	Involved in iron transport, also regulate some other metal transport.	Leads to integration of cell cycle with JAK-STAT3 and promote CRC [63].
ATP-citrate synthase (P53396)	Cleave citrate to OAA and Acetyl CoA.	Knockdown by siRNA leads to inhibition of chemoresistance to tested molecules [64].
Urokinase-type plasminogen activator (P00749)	Cleave plasminogen to plasmin.	miRNA suppressing its expression also reduces CRC cell proliferation and preogression [65].
Egl nine homolog 1 (Q9GZT9)	Cellular oxygen sensor.	The phosphorylation of PHD2 through mTOR and PP2A pathway modulate HIF1 alpha level and CRC cell survival during hypoxia.
Ferritin heavy chain (P02794)	Involved in iron storage in non toxic and readily available form.	The FHC level is reduced in lymph node metastasis associated with CRC poor prognosis in comparison to non lymph node metastasis CRC [66].
Flap endonuclease 1 (P39748)	It is a structure specific nuclease with 5'-3' exonuclease and 5'-flap endonuclease activity.	Genetic veriations is associated with several cancer including CRC [67].
DNA polymerase beta (P06746)	Involved in base excision repair.	It is a target for chemotherapeutic intervention of CRC [68].

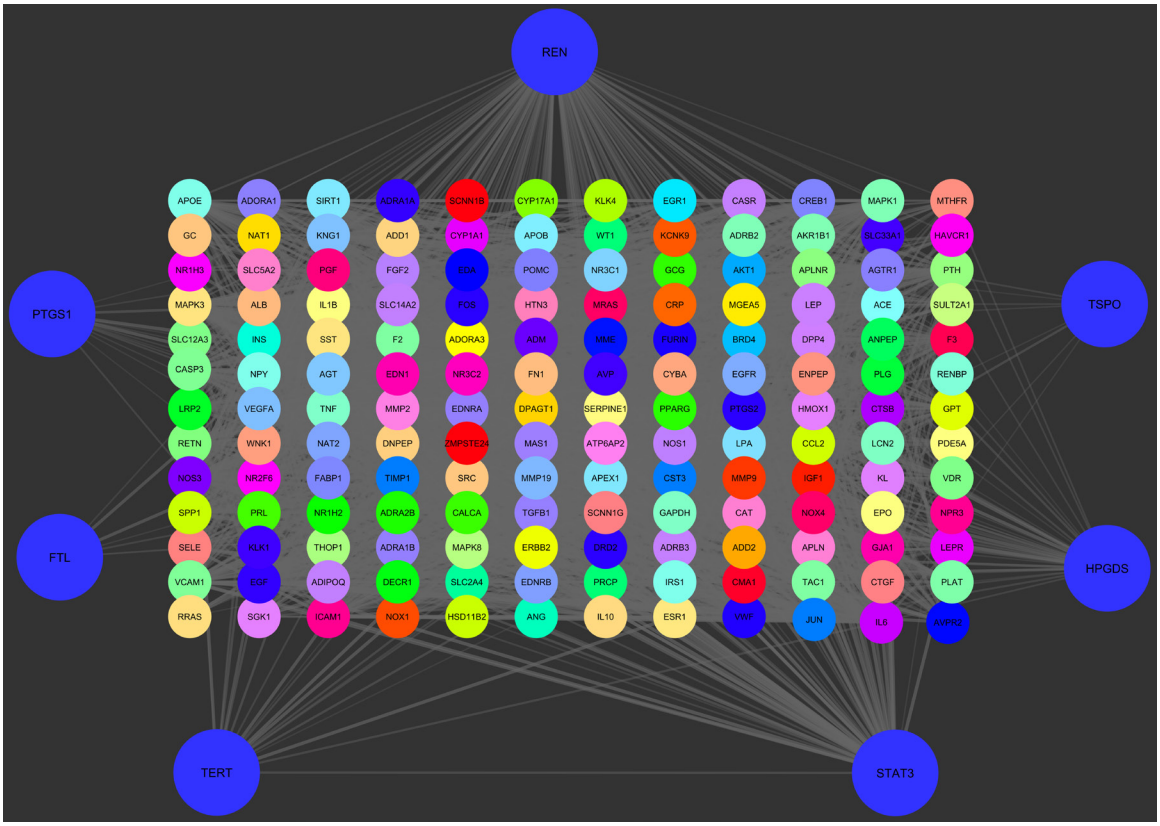

Figure 1. Protein-protein interaction map through STRING database for CRC associated heme targets and their direct interactors according to DisGeNet. The protoporphyrin IX (heme) targets are shown with large, blue color nodes, while STRING colors were used for their first neighbors.

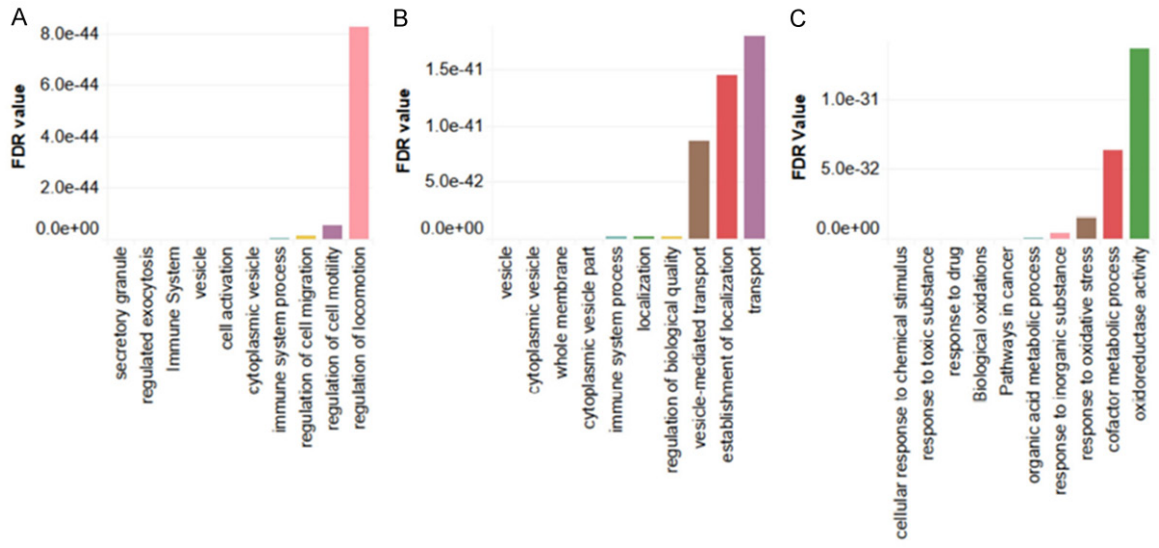

Figure 2. The STRING functional enrichment analysis of heme, nonheme targets and their interactors involved in CRC as per DisGeNet. Only top 10 hits are presented in the figure according to FDR value. A. Ferric citrate [nonheme]. B. Ferrous fumarate [nonheme]. C. Protoporphyrin IX [heme].

targets and their direct interactors are involved in CRC (**Figure 2**).

In order to understand more specific CRC-associated activity modulation by heme tar-

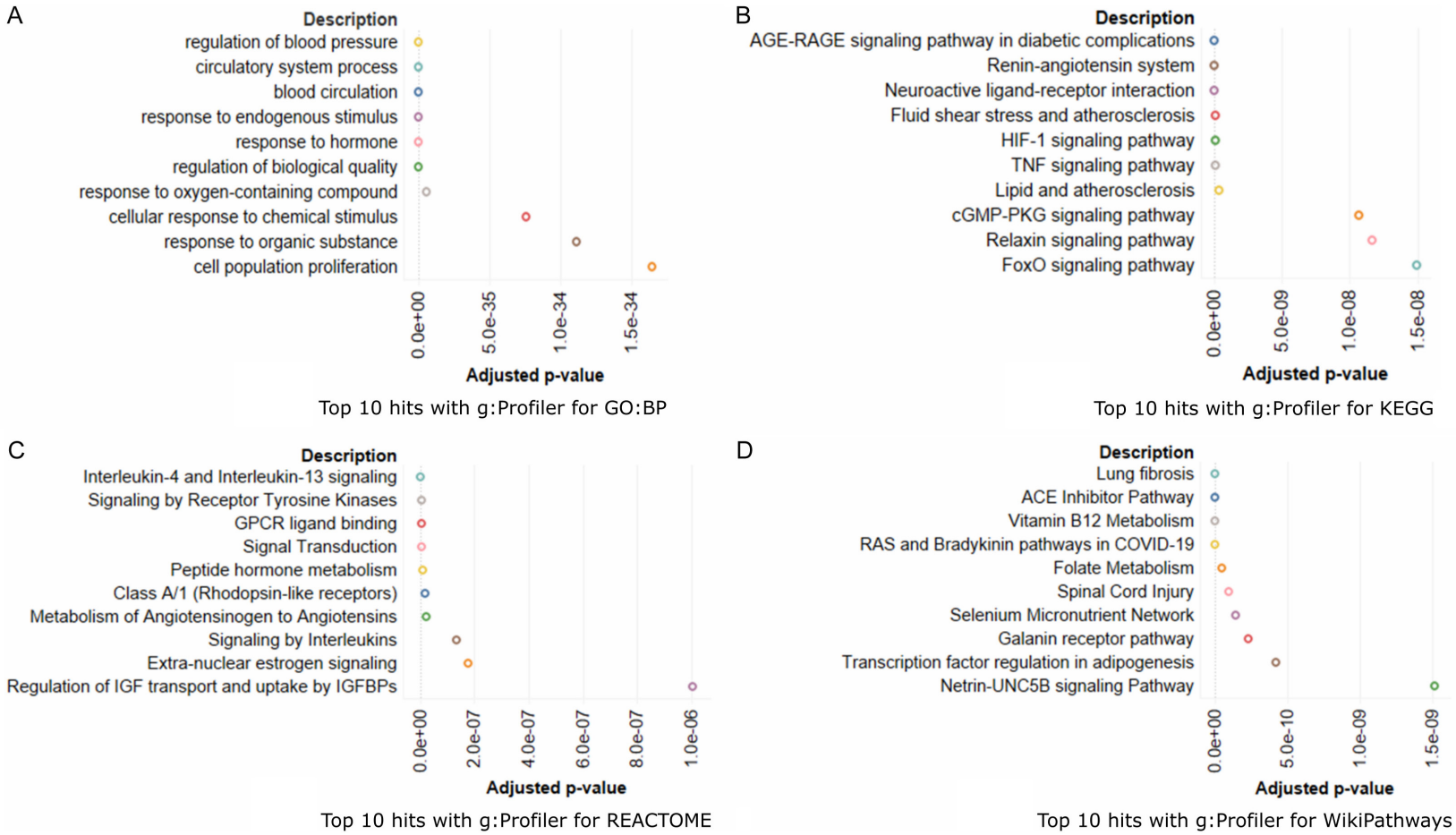

Figure 3. g:Profiler functional overrepresentation analysis of heme targets and their first neighbors involved in CRC as per DisGeNet. Only top 10 hits (on the basis of adjusted *P*-value) are presented for enrichment against (A) Gene Ontology:Biological Process, (B) KEGG, (C) REACTOME, and (D) Wikipathways.

gets, we separated all studied CRC associated heme targets and their first neighbors and analyzed them separately for functional enrichment. We used g:Profiler for functional overrepresentation analysis which is a well-known platform detecting statistically significant enriched terms against different databases like REACTOME, KEGG, Wikipathway, GO, etc. [30]. We found several cancer-associated processes and pathways enriched among top 10 processes using this approach (**Figure 3**). The gene ontology (biological process) based enrichment analysis indicated that heme targets are able to modulate cell population proliferation as indicated in **Figure 3**.

It was found that heme can influence interleukin-4 and interleukin-13 signaling as per enrichment with the REACTOME database. Several studies have found an important role of this pathway in a variety of cancer. IL-4 and IL-13 are immune-regulatory cytokines and share common receptors. The role of IL-13 and IL-4 in CRC has been discussed in several articles [31]. These cytokines and their receptors are considered as an important target for CRC management [32]. It is found that these cytokines can support CRC initiation and progression through NOX1 [33]. IL-4 is produced by Th2 cells and it is linked with abnormal STAT6 activation, resulting in CRC progression and metastasis. IL-4 downstream factor E2F1 promotes binding between specificity protein 3 (SP3) and STAT6 leading to its activation and subsequent tumorigenesis through E2F1/SP3/STAT6 signaling. Moreover, IL-4 is also involved in abnormal cell proliferation and inhibited apoptosis. The IL-4 is also involved in epithelial-mesenchymal transition by regulating epithelial and cytoplasmic markers, like E-cadherin and vimentin contributing to CRC. These roles of IL-4 in CRC tumorigenesis is already reviewed in the literature [34]. In addition, the role of IL-13 is also widely discussed in CRC tumorigenesis. IL-13 is linked with regulation of apoptosis through heme oxygenase 1 (HO-1). It is found that local IL-13 induction leads to increased HO-1 and antiapoptotic A20 leading to decreased intragraft apoptosis in rat model [35]. The role of IL-4 and 13 for colon cancer is already reviewed in detail in literature [36], in addition, iron metabolism is also known to be regulated by cytokines IL-4 and 13 [37]. The detection of modulation of these processes by heme and literature evidences about its apoptosis regulation potential by IL-13 indicate the involvement of IL-4/IL-13 signaling in heme mediated CRC and need experimental investigations. In addition, some studies have indicated the role of angiotensin converting enzyme (ACE) pathway in CRC. It has been recently proposed that ACE inhibitors or angiotensin receptor blockers are able to reduce the risk of CRC [38]. In addition, iron supplementation leads to ACE inhibitors associated cough through inhibition of NO synthase [39]. Our study predicted that heme targets are able to modulate ACE pathway, its inhibition or angiotensin metabolism. Therefore, it can be assumed that ACE pathway is an important target for heme mediated CRC etiology.

Hypoxia inducible factor 1 alpha (HIF 1α) is an important player mediating tumor response to hypoxia which affects invasiveness, aggressiveness and resistance of CRC [40]. The role of HIF in iron metabolism is already available in literature and reviewed in several articles [41]. Moreover, the prediction of HIF-1 signaling pathway modulation by heme targets during our study and its involvement in CRC indicate potential of this pathway in CRC etiology. TNF signaling pathway [42] and Foxo signaling pathway [43] are also known for modulating CRC etiology and prediction of these pathway modulations through heme targets indicate that these pathways must also be investigated experimentally in order to identify the mechanisms involved and subsequent preventive strategies for heme iron-mediated CRC etiology. Several mechanisms are already proposed for iron-mediated CRC, for instance, it has been discussed that iron concentration has an important effect on gut microbiota and probiotics can be used to achieve desired concentration of iron [44]. On the other hand, the role of microbiota and probiotics in CRC is already discussed [45-47] and therefore this link may also provide additional inputs for heme-mediated CRC development.

Although this study is computational in nature and computational methods have certain limitations, the use of well-known network pharmacology-based approach coupled with high quality targets indicates that these pathways must be considered in order to understand the role of heme in CRC etiology. The experimental

validation of all CRC associated pathways and processes can be a labor intensive task involving coordinated efforts of multiple laboratories which would involve a huge economic cost. Therefore, our study screened important targets with the potential to modulate CRC susceptibility and needs experimental investigations. The role of heme in CRC is already discussed in several articles. The colonic exposure of heme is linked with genotoxic and hyperproliferative effects through heme oxygenase and hydrogen peroxide as it has shown significantly increased cell proliferation in Caco-2 cells [48]. The heme oxygenase 1 expression is correlated with hypoxia inducible factor-1α [49], and over-expression of this signaling is also involved in poor prognosis in CRC patients [50].

Conclusion

These all indications give optimism for our findings and their potential in devising management strategies for heme mediated CRC. We are optimistic with our results and believe that these pathways can help find ways to manage CRC through modulation of heme mediated trigger. Though computational predictions must be seen with their limitations, current study identifies several pathways and targets for understanding heme iron mediated CRC etiology. The global efforts to unravel this enigma can be supported by these findings and the results can reduce time to understand complete picture of heme mediated CRC carcinogenesis.

Disclosure of conflict of interest

None.

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